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TITLE: Role of Ca^{++} Influx via Epidermal TRP Ion Channels

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14. ABSTRACT

To benefit military veterans with amputations who suffer skin problems on their amputation stumps, this proposal describes mechanistic studies to pave the way for novel methods of improving skin barrier function at the residual limb-prosthetic interface.
Signaling systems in skin will be modulated to increase barrier function, attenuate irritant dermatitis, and characterize the underlying signaling mechanisms so that they can become better targets for treatment.
Progress in year 1 of the funding period is described in this Annual Progress Report.
We gained all the necessary regulatory approvals from the Durham VA, Duke University IRB and the DoD, the latter late in the funding period. We set up experiments in primary skin cells for mechanical stress. Throughout the funding period we have modified and adjusted our customized apparatus for delivering mechanical stress to the cells. Measurement of skin capacitance works, as do assays to measure gene-expression. We have measured lactate dehydrogenase as a measure of cellular injury in mechanically-stressed human skin keratinocytes.

15. SUBJECT TERMS

TRP ion channel, epidermis, keratinocyte, mechanical stress, epithelial stress response

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Introduction

The objective of our proposal is to benefit military veterans with amputations who suffer skin problems on their amputation stumps. To fulfill the objective, this proposal describes mechanistic studies to pave the way for novel methods of improving skin barrier function at the residual limb-prosthetic interface.

Rather than attempting to enhance the functions of barrier-enhancing proteins, filaggrin (structural protein in upper epidermal layers) and aquaporins (water channel) by individual targeting, we thought it an attractive (and testable) idea, whether targeting of any of the epidermal transient receptor potential ion (TRP) ion channels could lead to **improved** barrier function and thus better moisturization, thus less irritation, injury and pain. This would be accomplished by affecting both, filaggrin and aquaporin. In keeping with our reasoning, recent insights on activation of TRPA1 and TRPV4 in the skin are supportive of our concept. However, an obstacle toward improved insight is that mammalian skin was not studied in response to mechanical stress in order to wear down the barrier, so that its more rational repair, by targeting specific signaling mechanisms of the epidermis, can be assessed.

Hypothesis

Therefore, the central hypothesis of our proposal is that Ca^{++} influx into epidermal keratinocytes – mediated via TRP ion channels that we intend to activate specifically - controls moisturization and barrier function of the skin by critically influencing filaggrin- and aquaporin function in a cell-autonomous manner, and that activation of these TRP channels can be helpful for repairing a compromised barrier which is caused by injurious mechanical stress.

Specific Aims

(1) to assess skin barrier function and moisturization parameters in response to modulation of TRPV1, 3 and 4 and TRPA1 in normal human skin as it is subjected to mechanical stress.

(2) to assess skin barrier function and moisturization in epidermal cultures derived from military veterans with amputations and stump skin irritation, and to determine the epidermis' response to activation of TRP channels.

Keywords

Amputation stump, irritant dermatitis, epidermis, skin barrier function, TRP channel, aquaporin (AQP) channel, UVB, UVA

Accomplishments

Approval for human experimentation

We applied for IRB approval, in the first place from the Durham VA. Our application had to be re-submitted in order to satisfy reviewers' comments. The revised application was approved. The Durham VA IRB approval was forwarded to the IRB of Duke University Medical Center. They also required revisions. We secured their approval as well.

The resulting IRB approvals of our institutions were forwarded to CDMRP-HRPO officials.

The required revisions were provided, by final approval of the Durham VA, on July 28, 2014. On August 8, 2014, the CDMRP-HRPO program officials approved of human experimentation, as proposed in our application, and as approved by Durham VA and Duke University IRBs.

Purchase of the skin capacitance meter for skin, both in culture and in-vivo

After due-diligence comparison, we purchased the device from NOVA Technology Corporation, Waltham MA. We purchased a capacitance meter with flexible probe, DPM9003BT. In addition to the device, we obtained an updated SINERGIE software package and a portable device for wireless data recording, so that recorded data can be e-mailed or uploaded onto our server via the internet. This platform will allow us to measure capacitance, which is a valid surrogate for moisturization, of skin both from human subjects in-vivo (non-invasively), and from cultured skin in culture. The specific design of the probe of our instrument supports this "dual purpose" measurements.

Human artificial skin

We obtained human EpiDermF (Mattek; IRB exempt) and grew it in culture. We adapted these cultured epithelial cells to the mechanical stress apparatus (Flexcell Devices). Whereas our skin cell culture is now functional and well-adapted, we are still facing obstacles with the mechanical stress device, to exert mechanical stress to our organotypic skin preparations.

We are currently calibrating these devices and ascertaining the needed minimal assay-to-assay variation when applying the mechanical stress.

Measurement of skin capacitance in culture

Using our NOVA capacitance meter with flexible probe, DPM9003BT, we established skin capacitance from human artificial skin in culture.

In three separate cultures, we measured initial capacitance of 105, 109 and 100 (arbitrary unit; AU). We then applied 5% glycerol (in serum-free culture media) for 1h, followed by glycerol-free culture media (5 minutes) and recorded an increase to 228, 235 and 218 AU.

Thus, we are confident that our skin impedance meter works properly, and that we are able to recap measurements in cultured artificial skin that reflect data as obtained from human subjects.

Based on this functional set-up, we applied specific TRPV4 activator GSK101, at a concentration of 10nM and 100nM. We measured capacitance 1h later. We were excited to find an increase in capacitance, from 102, 110, 114, 100, 118 AU (pre-application GSK101) to 160, 125, 188, 133, 194 (10nM), to 210, 195, 171, 162 and 145 (n=5 samples per experimental arm). We are currently modifying the application condition to mimic a topical application in a vehicle with increased viscosity in order to reduce variation, and to possibly accentuate the dose-response relationship. We also will test the 30, 120 and 360 min time points.

Activating TRPA1 with AITC at 50 and 100 µM did not appear to change capacitance, but from conducting the experiment – reduction of capacitance by 15-20%, high variation and lack of a dose-response-relationship, we suspect that

the dose could be too high, or that we are looking at other artefactual metrics. We will repeat at lower concentrations, and also use mustard oil.

Given our model working, we are currently getting into measuring the effects of activation of TRPV1 (olvanil, capsaicin) and of TRPV3 (camphor).

We are overall excited about the enhancing effect on barrier function of human skin that we observed with specific TRPV4 activation, and plan to conduct the remainder specific stimulations, the photostimulations next. In case we establish a certain profile of TRP-channel activation leading to enhancement of barrier, then we will also test whether specific TRPV4 blockers can render GSK101 (and eventually UVB, but awaiting positive results by experimental approach !) less potent at enhancement of barrier.

Stress-testing of human artificial skin

Figure: Bioreactor that does not build up sufficient strain. – We suspect insufficiently tight seals between the pistons that exert pressure on the cells and the pump system. Shown here is the set-up that permits 24 parallel stimulations, top-view (left), side-angle view (right).

EpiDermF (Mattek; IRB exempt) were grown in culture. We applied mechanical stress at strains of 0.3 and 0.4, using our modified Flexcell Bioreactor Compression system. – The Flexcell tension (see left-hand Figure) system did not reliably operate, especially in terms of experiment-to-experiment variation. Neither strain induced any physical damage in the EpiDermF specimens. We operated it at 1/sec sinusoidal strain for 1h, followed by 1h rest, for a total of 12h.

We have re-invigorated our attempts to make the Flexcell Bioreactor work, but did not succeed as we had hoped. Mechanical failures of the equipment prevented us from accomplishing higher strains, which we attempted to address, at one point together with the manufacturer. However, none of these troble-shooting attempts did deliver a breakthrough to this impasse.

We are currently custom-building an “upgraded” version of the Bioreactor Compression system that is designed to allow us to compress at high strain. This work is currently ongoing in the Duke machine shop.

Transcript levels in mechanically-stressed human artificial skin

EpiDermF was subjected to strains of 0.3 and 0.4. From these skin samples (n=4 mechanically stressed, n=5 baseline), we extracted RNA and subjected it to reverse transcriptase, followed by quantitative PCR.

We detected all TRP ion channels under study (TRPV1, TRPV3, TRPV4, TRPA1) as well as filaggrin. For the higher strain, 0.4, TRPV4 was moderately, but significantly increased (1.55-fold over control, $p<0.05$, t-test), the other channel transcripts were not significantly changed. Filaggrin appeared down-regulated, yet differences were not significant because of small experimental n with considerable variation.

For the lower strain, transcripts did not differ significantly.

Human experimentation

We finally addressed successfully the mindful issues brought up by the USAMRMC Office of Research Protections, Human Research Protection Office (HRPO). We complied with their requirement of having an appropriately qualified and unbiased study monitor in place, having a change to the consent form, mentioning that the study was sponsored by the DoD in the study protocol at specific passages within the narrative, and having to provide documentations of CITI training for the personnel who is in contact with subjects. All these requirements were addressed satisfactorily.

Impact

We view the pilot finding that selective activation of TRPV4 channels in skin can increase barrier function as a reportable outcome that has general as well as human health-related relevance.

Changes/Problems

The mechanical stress apparatus is not delivering sufficiently consistent mechanical strains. Efforts were made to rectify this problem, with involvement of experienced colleagues here at Duke as well as the manufacturer (Flexcell), but we ended up beginning to construct a new prototype of the apparatus because our attempts at repair were not met with success, despite dedicated and persistent approach.

With primary human skin, Matek EpidermF, there appears to be variation when applying compounds to the skin preparation. We are currently taking the approach to topically apply the compounds in vehicle with increased viscosity. We note that a vehicle-control will have to be included, but that this could also increase our chances of having the compound penetrate the epidermal layers at an improved, more efficient rate. We believe it will be worth to tackle this problem because it will be an issue – eventually – when considering treatment of live animals and human subjects.

Human Experimentation

Targets required: 15 military veterans and 15 healthy controls.

Approved by: Durham VA and Duke University IRB, CDMRP HRPO

Our efforts to facilitate recruitment became delayed because of change of clinics personnel at the Durham VA so that efficiency of reaching out to affected veterans became suboptimal

Products

As mentioned under “Impact”, we view the pilot finding that selective activation of TRPV4 channels in skin can increase barrier function as a reportable outcome that has general as well as human health-related relevance, at a level of non-incremental progress of knowledge.

Participants

Liedtke, Wolfgang MD, PhD; Associate Professor of Neurology – PI

Chen, Yong PhD; Senior Research Associate

Jin, Yingai; Research Technician

Special Reporting Requirements

Quad Chart attached.

Appendices

N/A

Role of Ca^{++} influx via epidermal TRP ion channels

W81XWH-13-1-0299 – Peer-reviewed orthopaedic research program: idea development award
OR120114

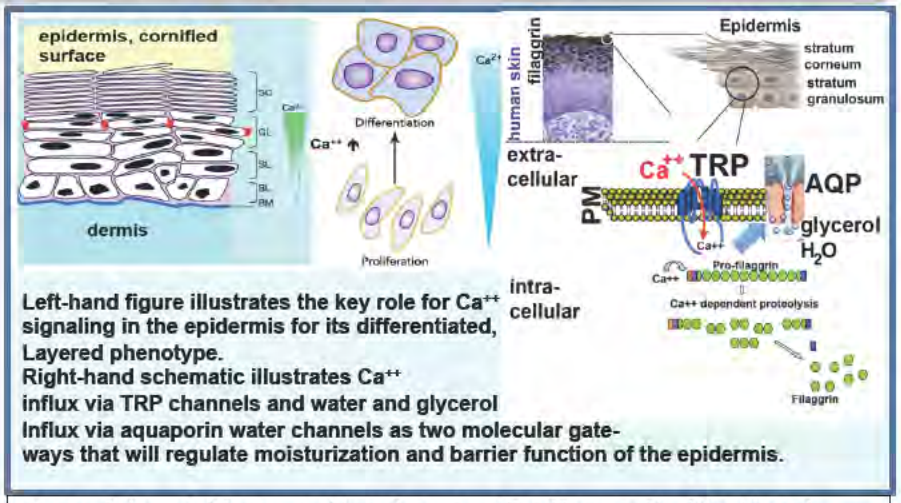
PI: Dr. Wolfgang Liedtke **Org:** Duke University, Durham NC **Award Amount: US$** 775,575.00 (total cost over 3y)

Study Aim(s)

(1) to assess skin barrier function and moisturization parameters in response to modulation of TRPV1, 3 and 4 and TRPA1 in normal human skin as it is subjected to mechanical stress.
(2) to assess skin barrier function and moisturization in epidermal cultures derived from military veterans with amputations and skin irritation of the prosthesis interface of the residual limb, and to determine the epidermis' response to activation of TRP channels.

Approach

We will specifically activate TRP ion channels in human skin keratinocytes and assess whether this has a beneficial effect on skin barrier formation and moisturization state. Human skin organotypic preparations will be used as a generic model (Aim 1), in addition skin biopsies and cultured keratinocytes from military veterans with skin irritation and dysfunctional barrier and impaired moisturization at the prosthesis interface of the residual limb (Aim 2).



Left-hand figure illustrates the key role for Ca^{++} signaling in the epidermis for its differentiated, Layered phenotype.
Right-hand schematic illustrates Ca^{++} influx via TRP channels and water and glycerol Influx via aquaporin water channels as two molecular gate-ways that will regulate moisturization and barrier function of the epidermis.

Accomplishment: (1) measured impedance in artificial human skin; (2) subjected artificial human skin to mechanical compression stress and measured gene expression for TRP channels and filaggrin; (3) moved human experimentation approval forward.

Timeline and Cost

Activities CY	14	15	16
Characterize mechanical stress response in cultured human skin			
Modulate mechanical stress response in cultured human skin			
Assess skin barrier function in epi-dermal cultures from mil veterans			
To define the response of cultured skin cells from military veterans to TRP channel activation			
Estimated Budget ($K)	**$166**	**$166**	**$166**

Updated: January 1, 2014

Goals/Milestones

CY14 & 15 Goals

☐ Characterize and modulate mechanical stress response in cultured human skin. – **Initiated and moved forward.**

☐ Assess and modulate skin barrier function in epidermal samples and cultures from military veterans with irritant dermatitis of their residual limb and healthy controls. – **Secured regulatory approval from the two institutions involved. DoD HRPO approval is on its way.**

CY16 Goal

☐ Assess and modulate skin barrier function in epidermal samples and cultures from military veterans with irritant dermatitis of their residual limb and healthy controls.

Comments/Challenges/Issues/Concerns

- Mechanical tension strain devices for cultured skin still problematic – addressing the problem currently; • secure final human expt. approval

Budget Expenditure to Date

Projected Expenditure: $187,218.20
Actual Expenditure: $187,218.20 ($121,415.26 direct cost)